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The Amino Acid Composition of Fish Collagen and Gelatin

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(*Received* 23 *July* 1956)

The major protein constituent of the skin, bones, swim bladder and scales of fishes resembles in many ways the more widely studied collagen of mammals. Thus it contains hydroxyproline and hydroxylysine, shows striated fibrils with the electron microscope (Borasky, 1950; Schmitt, Gross & Highberger, 1955; Damodaran, Sivaraman & Dhavalikar, 1956), and has characteristic wide-angle and narrow-angle X-ray diffraction patterns (Bear, 1952).

Amino acid analyses of fish collagens have been reported by Beveridge & Lucas (1944), who used mainly gravimetric methods, for isinglass from the swim bladder of hake (*Urophycis*); by Block, Horwith & Bolling (1949) for scales of herring (*Clupea*) and by Neuman (1949), who used microbiological-assay techniques, for halibut skin and for gelatin prepared from the scales of an unspecified fish. The recent study on the composition of elastoidin from the fin rays of the shark (*Carcharinus melanopterus*) by Damodaran *et al.* (1956), who used resin chromatography, has confirmed the results of wide-angle X-ray diffraction work (Astbury, 1938) in placing this unusual protein within the collagen group.

Interest in fish collagens has recently been stimulated by the suggestion that their reduced structural stability compared with mammalian collagens (shown by a lower range of shrinkage temperatures), is related to a lower content of hydroxyproline (Gustavson, 1953). Interchain hydrogen bonding between hydroxyl groups of hydroxyproline and backbone carbonyl groups was, on this basis, suggested as an important stabilizing feature of the collagen structure (Gustavson, 1955). This hypothesis has since been applied, without further proof, in interpreting details of the structure of collagen at two levels of size, in connexion with X-ray diffraction (Ramachandran, 1956) and electron-microscope techniques (Reed, Wood & Keech, 1956) respectively. A summary of recent work on the connective tissues of fishes is given by Hamoir (1955).

The present investigation provides information about the composition of collagen from a few species representing a zoologically wide range of fish types, members of all three surviving classes, Elasmobranchii, Actinopterygii and Crossopterygii (Young, 1950; Trewavas, White, Marshall & Tucker, 1955) being included. The experimental methods were

identical with those used in an earlier study of mammalian collagens (Eastoe, 1955), so that valid comparisons of small differences in composition can be made.

EXPERIMENTAL

Materials

Sturgeon swim-bladder collagen. Commercially available isinglass from the swim bladder of the Beluga sturgeon (*Acipenser huso huso*) was used as received. It is not known what earlier treatment this relatively pure product had undergone. The isinglass gave a 95% yield of clear gelatin when extracted under the conditions stated in Table 1.

Cod-bone gelatin. Cod (*Gadus morrhua*) vertebrae, which had been preserved in ice, were placed in 8% HCl for 3 days. The adhering muscle was pulled off and the demineralized bone was transferred to 10% acetic acid for 14 days. The bone collagen was repeatedly washed to remove acid and converted into gelatin by hot-water extraction (Table 1).

Shark-skin gelatin. Shark (probably *Selachus maximus*) skin was demineralized in 5% HCl for 5 days, cut into pieces and treated with saturated SO_2 solution for 20 days at 2°. Gelatin was extracted under the conditions given in Table 1.

Lung-fish collagen and gelatin. Skin of the Australian lung fish (*Neoceratodus*), which had been preserved by salting and drying, was soaked in water and the outer pigmented layer pulled off and discarded. The skin was cut into pieces, soaked in two changes of 10% NaCl for 24 hr. each, washed for 48 hr. in eight changes of water, dehydrated in acetone and ether, and finally air-dried. Moisture and ash contents of this and other proteins actually analysed are given in Table 1.

The lung-fish skin was soaked in 0·1 N-HCl for 18 hr., the acid was then neutralized by addition of NaOH soln. and the gelatin extracted in two stages (Table 1), giving 45 and 33% recoveries of the weight of skin respectively.

Methods

Hydrolysis. The proteins were hydrolysed in approx. 100 times their weight of 20% (w/w) HCl at 100° in sealed tubes for the times stated in Table 2.

Amino acid analysis, etc. Amino acid analyses, etc. were carried out by resin chromotography (Moore & Stein, 1951) exactly as described by Eastoe (1955), and the same corrections were applied. Hydroxyproline was determined by the method of Neuman & Logan (1950).

Kinematic viscosities. Kinematic viscosities of 6·67% (w/w) aqueous solutions of the gelatins with specified moisture and ash contents (Table 1) were measured at pH 6·0–6·5 according to B.S. 757:1944.

Rigidity determinations. Rigidity determinations were carried out on 6·67% gels, matured for 17 hr. at the specified temperatures, according to the method of Saunders & Ward (1953).

Shrinkage temperatures. Shrinkage temperatures were measured by visual observation of water-saturated samples freely suspended in toluene.

RESULTS

The amino acid contents of fish collagens and gelatins calculated on the weight of dry, ash-free protein are given in Table 2. The recovery of total

Table 1. *Moisture and ash contents of collagens and gelatins analysed; extraction conditions and physical properties of the gelatins*

Material		Water* (%)	Ash* (%)	Pretreatment (see text)	Extraction conditions: Time (hr.)	Temp.	pH	Kinematic viscosity (centistokes at 40°)	Rigidity† (dynes/cm.²)
Sturgeon swim bladder	Collagen	17·1	0·3	—	—	—	—	—	—
	Gelatin	—	—	None	3·0	60°	4·0	5·8	59 000
Cod bone	Gelatin	11·3	1·3	Acetic acid	3·0	60	4·4	5·3	28 000 at 0° 300 at 10° 0 at 14°
	Gelatin	—	—	Acetic acid	2·0	80	4·2	4·5	18 100 at 0° 0 at 10°
Shark skin	Gelatin	—	—	HCl, SO_2	3·0	60	4·0	4·3	45 000
	Gelatin	13·3	2·8	HCl, SO_2	3·0	80	4·0	2·5	14 000
Lung-fish skin	Collagen	18·3	0·14	—	—	—	—	—	—
	Gelatin	14·1	5·0	HCl	5·5	65	5·5	‡	56 000
	Gelatin	—	—	HCl	5·5	80	3·5	‡	8 400

* Moisture and ash values are given only for those proteins for which the amino acid analyses are reported.
† At 10° unless otherwise specified.
‡ Not determined.

nitrogen ranged from 97·6 to 102·5 %, the extreme values not greatly exceeding the expected experimental error of ± 2 %. The recovery by weight ranged from 93·7 % for collagen of lung-fish skin to 99·2 % for sturgeon swim bladder. These figures were lower than those for mammalian materials, and suggested a higher content of non-nitrogenous components in some of the fish proteins.

Traces of cysteic acid and possibly cystine were detected in the hydrolysates but amounts were such as to suggest that the cystine content did not exceed 0·1 %. Tryptophan was not determined.

Some physical properties of the gelatins derived from fish collagens are summarized in Table 1. The viscosity values are only slightly below those of the mammalian gelatins investigated previously (Eastoe, 1955). This suggests that the fish and mammalian gelatins are of the same order of average molecular size.

The rigidities of the sturgeon, shark and lung-fish gelatins were somewhat lower than those of mammalian gelatins. It is possible that these reduced values arose through failure to find the optimum conditions for extraction of gelatin from the fish materials, although it is thought that there was probably a definite intrinsic difference. Pretreatment and extraction conditions had been adjusted for each material in an attempt to produce gelatin with minimum degradation, but the effect of the various variables was not known with the same certainty as for mammalian gelatin, the extraction of which has been widely studied.

The rigidity of the material extracted from cod bone was markedly lower than for the other fish gelatins. At 10° a 6·67 % gel gave a low value, so that the measurement was repeated at 0°. The melting point of this gel was 12°, compared with 30° for mammalian gelatin. Gelatins from cod skin and swim bladder have also been found to have comparable, greatly diminished gelling properties, 6·67 % gels melting at 13° (Dr A. Courts, private communication).

Table 2. *Amino acid composition of fish collagens and gelatins*

Values are given as g. of amino acid/100 g. (*G*), and moles of amino acid/10^5 g. (*M*) of dry ash-free protein.

	Sturgeon swim-bladder collagen		Cod-bone gelatin		Shark-skin gelatin		Lung-fish skin			
							Collagen		Gelatin	
Duration of hydrolysis (hr.) ...	24		24		24		48		48	
	G	*M*	*G*	*M*	*G*	*M*	*G*	*M*	*G*	*M*
Alanine	11·6	130	10·4	115	11·2	126	11·7	131	11·9	134
Glycine	27·7	369	28·2	376	26·5	353	24·0	319	26·1	348
Valine	2·31	19·7	2·32	19·7	2·71	23·2	2·56	21·8	2·21	18·9
Leucine	2·55	19·4	3·26	24·8	3·32	25·3	3·37	25·8	2·75	21·0
Isoleucine	1·65	12·5	1·64	12·5	2·71	20·6	1·64	12·5	1·34	10·2
Proline	12·8	111·7	12·4	107·8	13·9	120·2	14·8	129·0	15·8	137·2
Phenylalanine	2·53	15·4	2·04	12·3	2·43	14·7	2·60	15·7	2·41	14·6
Tyrosine	0·46	2·6	0·63	3·6	0·26	1·5	0·19	1·1	0·14	0·8
Serine*	5·8	55·2	7·9	75·3	5·0	47·2	4·71	44·8	4·71	44·8
Threonine*	3·79	31·9	3·22	25·7	3·24	27·3	3·18	26·7	3·01	25·2
Cystine†	—	—	—	—	—	—	—	—	—	—
Methionine‡	1·43	9·6	2·26	15·1	1·59	10·6	0·59	4·1	0·54	3·6
Arginine	10·0	57·3	9·1	52·3	9·3	53·3	9·1	52·2	9·9	56·9
Histidine	0·83	5·3	1·24	8·0	1·26	7·8	0·80	5·2	0·75	4·8
Lysine	3·46	23·8	3·66	25·1	3·76	25·7	3·63	24·8	3·63	25·0
Aspartic acid	6·9	51·9	7·5	55·9	6·0	45·1	6·6	49·8	6·2	46·7
Glutamic acid	11·4	77·1	11·4	77·7	10·3	69·7	11·9	80·8	11·9	81·3
Hydroxyproline	11·8	89·6	8·3	63·2	10·9	83·2	9·8	74·8	10·8	83·2
Hydroxylysine	1·90	11·7	1·42	8·8	0·82	5·0	0·88	5·4	1·08	6·7
Total	118·9	1093·7	116·9	1078·8	115·2	1059·4	112·05	1024·5	115·17	1062·9
Amide N§	0·63	44·9	0·67	48·0	0·43	31·1	0·67	47·9	0·63	45·0
Total N	18·54		18·38		18·21		18·24		18·21	
Mean wt. of residue	90·7		90·3		90·8		91·35		90·37	
Z‖	119·9		119·9		119·8		119·7		119·9	
Recovery by wt. (%)	99·2		97·5		96·2		93·7		96·1	
Recovery of N (%)	102·5		101·7		99·9		97·6		101·2	

* Corrected for decomposition during hydrolysis (see Eastoe, 1955).
† Trace (see text).
‡ Sum of methionine and methionine sulphoxide peaks.
§ Corrected for ammonia formed by decomposition of serine and threonine.
‖ Chibnall (1942).

DISCUSSION

Comparison with previously published results

A number of analyses of fish connective-tissue proteins have been made by earlier workers, but comparison is complicated by the wide range of types examined and the consequent probability of variation between collagens from zoologically remote species. In Table 3, the available data are summarized for glycine and those amino acids present in substantially different proportions in fish collagen compared with mammalian material.

Almost all of the results support earlier findings that, in general, the imino acids proline and hydroxyproline (Gustavson, 1955) are reduced in fish, compared with mammalian collagen, whereas serine (Neuman, 1949), threonine and methionine (Beveridge & Lucas, 1944) are increased in fish collagen. Large amounts of glycine are reported throughout except by Beveridge & Lucas (1944).

The hydroxyproline value obtained for halibut skin by Neuman & Logan (1950) and the present values obtained by the same method appear to be systematically higher than those of Gustavson (1955) for cod- and pike-skin collagens, which are based on Kjeldahl nitrogen determinations of hydroxyproline separated on Dowex 50 resin. This discrepancy, the reason for which is not clear, makes detailed comparison of hydroxyproline data difficult. The very low value for hydroxyproline in hake swim bladder (Beveridge & Lucas, 1944) is suspect, since it is stated by these authors to be a minimal one, possibly much below the true value. The figure for shark elastoidin was obtained by the ninhydrin method.

Characteristics of the composition of fish collagens

Fish collagens and gelatins resemble those of mammals (Eastoe, 1955) in containing the same amino acids in, broadly speaking, similar proportions. A rather wider range of composition is observed in fishes than in mammals, which is not surprising in view of the greater evolutionary age of fishes. As a group they have been exposed to wide environmental changes during the long period of existence, and their consequent adaptation has led to a great variety of types. Since some of the more primitive types have fortuitously survived relatively unchanged, alongside those more recently evolved, a wide range of material of evolutionary interest is available for biochemical work.

The diminished content of imino acids in fish collagens compared with those of mammals is well marked. Hydroxyproline, in particular, is notably low, although showing considerable variation among fish species. The lowest value in the present

Table 3. *Comparison of present values for certain amino acids with reported values*

Values refer to amino acid N as a percentage of total N.

Reference	Eastoe (1955)	Present study					Neuman (1949)	Block *et al.* (1949)	Beveridge & Lucas (1944)	Damodaran *et al.* (1956)	Gustavson (1955)	
Material	Ox-bone collagen	Sturgeon swim bladder	Cod-bone gelatin	Shark-skin gelatin	Lung-fish skin gelatin		Halibut skin	Herring scales	Hake swim bladder	Shark* elastoidin	Cod skin	Pike skin
Glycine	27·5	27·9	28·7	27·2	26·7		30·0	24·0	9·8	27·0	—	—
Proline	10·1	8·4	8·2	9·3	10·5		9·1	6·5	11·9	9·2	7·2	8·8
Hydroxyproline	8·2†	6·8†	4·8†	6·4†	6·4†		5·3†	—	2·8	5·4	3·4	4·6
Serine	2·9	4·2	5·8	3·6	3·4		4·3	6·2	3·0	2·5	—	—
Threonine	1·5	2·4	2·0	2·1	1·9		2·3	2·1	2·1	1·6	—	—
Methionine	0·4	0·7	1·2	0·8	0·3		1·2	1·7	1·4	0·96	—	—
Shrinkage temp.	65°	50°	40°	53°	63°		40° ‡	—	—	63–64°	40°	55°

* Contained 3·16% of tyrosine N.

† Determined by method of Neuman & Logan (1950).

‡ Gustavson (1953).

study is 4·8 % (based on nitrogen content) for cod-bone gelatin, which may be compared with 8·2 % in ox-bone collagen.

Increased amounts of the aliphatic hydroxy amino acids serine and threonine were present in all the fish proteins examined, compared with the amounts in mammalian collagen. The hydroxylysine content was substantially increased in two of the fish materials. The increase of hydroxyl groups in the molecule, owing to the larger numbers of aliphatic hydroxy amino acid residues in fishes, was approximately equal to the loss of groups from the diminished number of hydroxyproline residues. This balancing effect results in the collagen molecule containing approximately the same proportion of hydroxyl groups in fishes and mammals.

The methionine content is very variable in fish collagens, ranging from nearly three times the mammalian figure in cod to some 60 % of it in the lung fish. Glycine and alanine, both amino acids present in large quantities in collagen, occur in almost identical amounts in fishes and mammals. In the absence of satisfactory physical criteria of purity for a fibrous protein such as fish collagen, the high values for these two amino acids suggest that the preparations were not grossly contaminated with protein impurities, which the hydroxyproline values taken alone might otherwise imply. A somewhat enhanced arginine content, offset by diminished lysine, is apparent in fish compared with mammalian collagen. The histidine contents of cod and shark gelatins are somewhat higher than the remaining fish materials, which have values closely similar to mammalian collagen. Thus the collagen of both mammals and fishes is notably low in histidine.

In all the fish collagens and acid-processed gelatins derived from them, the number of free carboxyl groups (aspartic acid + glutamic acid − total amide) is nearly equal to the sum of the basic residues (arginine + lysine + histidine), excluding hydroxylysine. It is particularly interesting that this relationship still holds for shark gelatin, where the carboxylic acids are diminished in amount compared with the other species, but this is offset by a correspondingly low amide content. The same relationship holds for mammalian collagens and may be regarded as an unusual characteristic of this group of proteins. The presence of hydroxylysine upsets this balance, resulting in a slight preponderence of basic groups in the molecule, which accounts for the iso-ionic point of acid-processed gelatins, and presumably the parent collagens, lying well to the alkaline side of neutrality (pH 9·0–9·5).

The effect of converting fish collagen into gelatin was to increase further those amino acids present in large amounts, with a corresponding reduction in those present in small quantities. These trends had already been noted in mammalian collagen (Eastoe, 1955).

The greatest departure from the mammalian composition is found in cod (*Gadus morrhua*), which is a member of the order Teleostei containing the most recently developed, specialized and currently successful of the actinopterygian fishes. The sturgeon (*Acipenser huso huso*), a comparatively unchanged member of the primitive palaeoniscid stock of the Actinopterygia, showed less extreme values for hydroxyproline, serine and threonine.

The shark, a representative of a separate class, Elasmobranchii, showed no great variation in composition from the sturgeon. The Australian lung fish (*Neoceratodus*) is particularly interesting in possessing a skin with a high shrinkage temperature, close to that of mammalian skins. The composition was fairly close to that of mammals with slightly enhanced serine and threonine and reduced hydroxyproline. The lung fish, as one of the four surviving species of the class Crossopterygii, is generally supposed to be more closely related to land vertebrates than any other living fish (Young, 1950).

Much labour would be needed for a systematic investigation of the composition of collagen in the many types of fishes; the present study gives some indication of the variations to be expected among the main groups.

Stability of intermolecular structure of collagen

Gustavson (1953, 1955) has pointed out the lower degree of intermolecular stabilization of fish (cod-skin) collagen, compared with mammalian collagen. Fish collagens are readily gelatinized, easily degraded and solubilized by acids and alkalis, and are attacked by trypsin and dissolved by anionic detergents. Mammalian collagens show much greater resistance under these conditions and have a higher shrinkage temperature. The ready conversion of cod skin and swim bladder into soluble degradation products has been confirmed in this Laboratory and the low rigidity of gelatin from cod bone (Table 1) is possibly a further manifestation of the low intermolecular stability of the parent collagen. Saunders & Ward (1955) have shown that the rigidity of different fractions of mammalian gelatin is independent of viscosity and also probably of molecular weight, above a limiting value. They have suggested that another factor, probably of a specific structural nature, controls the rigidity of gelatin gels. Ability to form extensively hydrogen-bonded systems may control both the interchain stability of collagen and the gelling of gelatin.

Gustavson (1955) has classified collagens of fish skins into two distinct groups on the basis of shrinkage temperature (T_s): (1) cold- and deep-water fishes (T_s 37–45°) and (2) warm- and surface-water fishes (T_s 50–57°). Hamoir (1955) considered this

division too rigid and suggested that there is a regular transition of shrinkage temperature from 35 to 59°. Shrinkage temperature does not appear to be related to the broad zoological classification, but rather to the habitat of a species. Thus among the Teleostei, the cod has a shrinkage temperature of 40°, whereas that of the pike (*Esox lucius*) is 55°.

Of the four species examined here, the cod alone had a shrinkage temperature near the lower end of the scale (Table 3), sturgeon and shark had much higher values, which placed them among Gustavson's pelagic fishes, and lung fish had the exceptionally high value of 63°. The marked divergence of the composition of cod gelatin from the mammalian type has already been pointed out, as well as its diminished rigidity. The remaining three fish types have hydroxyproline contents intermediate between cod and mammals, the shrinkage temperatures (with the exception of lung fish) and gel rigidities being also intermediate. Among these collagens of individual species, however, the physical properties do not run parallel to the hydroxyproline contents.

Although it seems probable that the intermolecular stability of the polypeptide chains in collagen and gelatin gels increases with hydroxyproline content, the existence of a specific hydroxyproline hydrogen bond remains somewhat speculative. It is questionable whether a single bond, having an energy of only a few kcal./mole, such as a hydrogen bond, would be of much importance acting alone in proteins where extensive backbone hydrogen bonding could occur. Hydroxyproline hydrogen bonds would have a considerable total energy for a collagen molecule of molecular weight 350 000. This quantity of energy might be important, although its very wide distribution suggests that for its effective utilization the hydroxyproline bonds would need to occupy key positions in a systematically arranged set of backbone bonds. It is also possible that other factors influence the hydrothermal stability of proteins of the collagen type. Damodaran *et al.* (1956) have pointed out the probable effect of the high contents of tyrosine and cystine on the hydrothermal properties of the rather specialized collagen in shark elastoidin. The abnormally high shrinkage temperature of lung-fish gelatin cannot be explained entirely in terms of its hydroxyproline content.

SUMMARY

1. The amino acid compositions of collagens and derived gelatins from sturgeon, cod, the shark (*Selachus maximus*) and the Australian lung fish have been determined.

2. These fish collagens show a similar amino acid distribution to mammalian collagen, with decreased amounts of proline and hydroxyproline, and increased serine, threonine and, in some cases, methionine and hydroxylysine.

3. Gelatin prepared from cod bone has a very low rigidity at 10°, whereas sturgeon, shark and lung-fish collagens give rise to gelatins having rigidities of the same order as, but probably somewhat below, those of mammalian gelatins.

4. In general, the shrinkage temperature of the collagen and gel properties of extracted gelatin decrease with decreasing hydroxyproline content. Certain departures in detail from this behaviour indicate that other unknown features of composition may influence the stability of linkages between polypeptide chains.

5. Variations in the properties and composition of fish collagens seem to be related to the water temperature of the normal habitat, rather than to considerations of broad zoological classification.

I am grateful to the Secretary of the Department of Harbours and Marine, Brisbane, Australia, for kindly providing the lung-fish specimen, and to James Vickers and Co., who supplied the sturgeon swim bladder. The rigidity experiments were carried out by Mr P. R. Saunders and the shrinkage temperature measurements by Dr A. Courts, to whom I am also indebted for much valuable discussion. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

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